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Abstract: **OBJECTIVES** The microbial composition of peri-implantitis-associated biofilms may resemble that of periodontitis, with some distinctive differences, as identified by various conventional or molecular detection methods. Yet, the complete microbiome of peri-implantitis awaits further characterization. The present clinical study was undertaken with the aim to investigate the association of Spirochaetes, and the more recently identified phylum Synergistetes, with peri-implantitis. **MATERIALS AND METHODS** Submucosal biofilms were obtained from single sites of patients with peri-implantitis ($n = 43$) or individuals with peri-implant health ($n = 41$). The samples were analysed by fluorescence in situ hybridization (FISH) and epifluorescence microscopy, using 16S rRNA-based oligonucleotide probes for Synergistetes cluster A, subclusters A1 and A2, and Treponema groups I-III and IV. **RESULTS** Treponema group IV was barely detectable, whereas Treponema groups I-III were detected at low prevalence in health, but their prevalence and numbers were significantly increased in peri-implantitis by 48% and 2.4-log, respectively. Synergistetes cluster A was detected in half of the healthy sites, and its prevalence and numbers were significantly increased in peri-implantitis by 30% and 2.5-log, respectively. No quantitative differences were found between Synergistetes subclusters A1 and A2 numbers, as both increased by 2.8-log. Synergistetes cluster A displayed strong correlations with several clinical peri-implant parameters, but Treponema groups I-III only with probing pocket depth. **CONCLUSION** The present clinical cross-sectional study demonstrates that Spirochaetes of the Treponema groups I-III, but not group IV, and Synergistetes of the cluster A are highly associated with peri-implantitis. Synergistetes cluster A appears to display a stronger association with peri-implantitis than Spirochaetes.

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Clinical association of *Spirochaetes* and *Synergistetes* with peri-implantitis

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Abstract

Objectives: The microbial composition of peri-implantitis-associated biofilms may resemble that of periodontitis, with some distinctive differences, as identified by various conventional or molecular detection methods. Yet, the complete microbiome of peri-implantitis awaits further characterization. The present clinical study was undertaken with the aim to investigate the association of *Spirochaetes*, and the more recently identified phylum *Synergistetes*, with peri-implantitis. **Materials and Methods:** Submucosal biofilms were obtained from single sites of patients with peri-implantitis (n=43) or individuals with peri-implant health (n=41). The samples were analyzed by fluorescence *in situ* hybridisation (FISH) and epifluorescence microscopy, using 16S rRNA-based oligonucleotide probes for *Synergistetes* cluster A, sub-clusters A1 and A2, and *Treponema* groups I-III and IV. **Results:** *Treponema* group IV was barely detectable, whereas *Treponema* groups I-III were detected at low prevalence in health, but their prevalence and numbers were significantly increased in peri-implantitis by 48% and 2.4-log, respectively. *Synergistetes* cluster A was detected in half of the healthy sites, and its prevalence and numbers were significantly increased in peri-implantitis by 30% and 2.5-log, respectively. No quantitative differences were found between *Synergistetes* sub-clusters A1 and A2 numbers, as both increased by 2.8-log. *Synergistetes* cluster A displayed strong correlations with several clinical peri-implant parameters, but *Treponema* groups I-III only with probing pocket depth. **Conclusion:** The present clinical cross-sectional study demonstrates that *Spirochaetes* of the *Treponema* groups I-III, but not group IV, and *Synergistetes* of the cluster A are highly associated with peri-implantitis. *Synergistetes* cluster A appears to display a stronger association with peri-implantitis than *Spirochaetes*.

Keywords: peri-implantitis; dental implants; microbiology; fluorescence *in situ* hybridization; *Spirochaetes*; *Synergistetes*.

Running head: *Spirochaetes* and *Synergistetes* in peri-implantitis

Introduction

Although it is well established that the microbiological features of peri-implantitis closely resemble those of periodontitis (Mombelli & Decaillet 2011), novel molecular microbiological methods are shedding light to some distinctive differences between the two disease forms (Belibasakis 2014, Belibasakis, et al. 2015, Charalampakis & Belibasakis 2015, Dabdoub, et al. 2013, Faveri, et al. 2015, Kumar, et al. 2012). Historically, the lack of appropriate protocols to cultivate and characterize at least half of the oral taxa has also hampered our understanding of the microbiota associated with peri-implantitis. Yet, with the use of molecular detection methods, we are becoming increasingly able in bridging this knowledge gap. Among such is the 16S rRNA-based fluorescence *in situ* hybridisation (FISH), which utilizes epifluorescence microscopy to identify non-cultivable taxa at the genus or species level (Gmur, et al. 2000, Wecke, et al. 2000). This approach has been successfully used in the characterization of the periodontitis-associated residing in subgingival biofilms (Ammann, et al. 2013, Drescher, et al. 2010, Vartoukian, et al. 2009, Zijnge, et al. 2010).

Among the largely uncultivable taxa to this date are *Spirochaetes* and *Synergistetes*, which are two of the 13 different phyla identified in the Human Oral Microbiome Database (HOMD) (Dewhirst, et al. 2010). A large proportion of *Spirochaetes* belong to the genus *Treponema*. Treponemes are unicellular Gram-negative spiral-shaped anaerobic rods of varying length. There is a well-established association between their presence in subgingival biofilms and chronic or aggressive

periodontitis (Dewhirst, et al. 2000, Haffajee, et al. 2006, Moter, et al. 1998), whereas there is also evidence of their involvement in peri-implantitis (Papaioannou, et al. 1995, Renvert, et al. 2007).

The bacterial phylum *Synergistetes* consists of Gram-negative anaerobic curved rods. They can be found as part of the healthy oral microbiota (Horz, et al. 2006, Vartoukian, et al. 2007), and their particular association with oral disease has been demonstrated in periodontitis (Belibasakis, et al. 2013, Vartoukian, et al. 2009, You, et al. 2013) necrotizing gingivitis (Baumgartner, et al. 2012) and endodontic infections (Fernandes Cdo, et al. 2014). To date, the human oral *Synergistetes* are divided principally in clusters A and B (Vartoukian, et al. 2009), with cluster A being more associated with oral disease than cluster B (Belibasakis 2014, You, et al. 2013), and exhibiting phylogenetically a more distinctive division in sub-clusters A1 and A2 (Baumgartner, et al. 2012).

While there is increasing evidence of the presence of *Spirochaetes* in peri-implantitis (Koyanagi, et al. 2013, Zhuang, et al. 2014), the presence of *Synergistetes* in the disease has barely been studied (Koyanagi, et al. 2010, Maruyama, et al. 2014). Therefore, by using FISH in combination with epifluorescence microscopy, the primary aim of this clinical study was to evaluate prevalence and numbers of known human oral *Synergistetes* and *Spirochetes* in submucosal biofilm of patients with peri-implantitis or individuals with peri-implant health. A further aim was to investigate the association between the levels of these taxa and clinical parameters of the severity of peri-implant disease. It is hypothesized that higher levels of *Synergistetes* and *Spirochaetes* will be identified in submucosal biofilm samples of patients with peri-implantitis, than individuals with peri-implant health.

Materials and Methods

Patient recruitment

This study was approved by the Ethical Review Committee of the Canton of Zürich, Switzerland (KEK-Nr: 2011-0159), and was conducted in accordance with the guidelines of the world Medical Association Declaration of Helsinki. Patients were recruited as outpatients who were referred by private practitioners for the diagnosis and treatment of peri-implant health associated problems at the Interdisciplinary Peri-implantitis Unit, Center of Dental Medicine at the University of Zürich. Potential participants were informed about the voluntariness of their participation and aim of the study. Inclusion criteria involved good overall medical health as evidenced by the medical history, at least 18 years of age, and willingness to participate in the study. Criteria for exclusion were periodontal or peri-implant treatment within the past 12 months, intake of systemic antibiotics within the past 6 months and pregnancy or lactation. The selection of patients was based on radiographic evaluation of the marginal bone level, clinical signs of inflammation and/or presence of pus. For inclusion into the peri-implantitis group, the intraoral radiographic examination demonstrated at least one implant with post-insertion radiographic marginal bone loss of 2.0 mm or greater mesially or distally, over a period of one year. The control group included implants with a healthy peri-implant status (absence of pus or visible/detectable radiographic bone loss), which were functionally loaded for at least one year. The demographic parameters of gender and age were recorded. Clinical parameters included diagnosis of peri-implantitis, implant age (time from installation), suppuration, radiographic bone loss, width of the keratinized mucosa, as well as plaque index (PI), bleeding on probing (BOP) and probing pocket depth

(PPD). The latter three parameters were measured at 6 sites per implant. All subjects provided written and informed consent for sample collection.

Submucosal biofilm sample collection

Submucosal biofilm samples were collected from sites of patients with either peri-implantitis, or healthy peri-implant sites of healthy subjects. In cases with multiple implants, one single implant was randomly sampled per patient. The samples were taken from the mesio-vestibular pocket of the implant. For this, the supramucosal areas of the implant and supra-structure were isolated from cheek and tongue using cotton rolls, and air-dried. Supramucosal biofilm was carefully removed with a cotton pellet, and the submucosal biofilm samples were obtained with a Gracey curette (Deppeler, Rolle, Switzerland). The biofilm sample collected by the first stroke was transferred to a micro-centrifuge tube containing 0.1 ml of RNase-free TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). The biofilm sample collected from the second to the fifth stroke was transferred to a another micro-centrifuge tube with 0.1 ml of 0.9% NaCl containing 1:500 RNase Inhibitors (Sigma Aldrich, Buchs, Switzerland), and stored at -80°C until further use. This content of this latter tube was further processed for the microbiological analysis by FISH performed in this study, as detailed in the next section.

Analysis biofilm samples by fluorescent *in situ* hybridization (FISH)

The FISH analysis of the biofilm samples was performed according to previously described protocols (Baumgartner, et al. 2012). Briefly, after thawing, the samples were vortexed for 1 min and diluted 1:10 in coating buffer (0.9% NaCl, 0.02% NaN₃, 2.5 x 10⁻⁴ % hexadecyltrimethylammonium bromide). Ten µl of the suspensions were

then spotted on individual 18- or 24-well epoxy coated ADCELL multi-well slides, with a well-diameter of 4 mm (Cel-Line, Erie Scientific Company, Portsmouth, NH, USA). Following air-drying, the slides were fixed with 4% paraformaldehyde (PFA) at +4°C, for 20 min, and each well was there after covered by 9 µl of Denhardt's solution (Fluka, Buchs, Switzerland; diluted 1:50 in PBS) with 1:500 Protect RNA RNase inhibitor (Sigma Aldrich, Buchs, Switzerland), and incubated at 37 °C, for 1 h. For the hybridization step, five specific oligonucleotide rRNA probes were used for *Synergistetes* cluster A (Fernandes Cdo, et al. 2014, Vartoukian, et al. 2009, Zijnge, et al. 2010), *Synergistetes* sub-cluster A1, *Synergistetes* sub-cluster A2 (Baumgartner, et al. 2012), *Treponema* cluster 1 (Zijnge, et al. 2010) covering multiple oral treponemes of the previously determined groups I-III (Choi, et al. 1996) (including *Treponema denticola*, *Treponema vincentii*, *Treponema medium*, *Treponema parvum*), and *Treponema* group IV (including *Treponema lecithinolyticum* and *Treponema maltophilum*) (Choi, et al. 1996, Guggenheim, et al. 2009) and one universal probe, which identifies most eubacteria (Amann, et al. 1995). The oligonucleotide sequences of the rRNA probes labelled with Cy3 or 6-FAM (Microsynth, Balgach, Switzerland), their targeted taxa, and the respective publications in which they were validated are listed in Table 1. The final probe concentrations used for FISH were 5 ng/µl for Cy3 conjugates and 20 ng/µl for FAM conjugates, in the presence of 35% or 40% formamide. The FISH-stained samples were thereafter visualized by an Olympus BX60 fluorescence microscope (Olympus Optical, Volketswil, Switzerland). For the quantitative estimation of the bacterial numbers, at least 10 viewing fields per well were counted at 100x magnification. The total number of positive bacteria was calculated per sample, as previously described (Baumgartner, et al. 2012, Gmur & Thurnheer 2002).

Statistical analysis

The differences in clinical parameters (patient age, implant age, bone loss, BOP, PI, PPD, keratinized mucosa) between the two diagnosis groups was statistically analysed by the Mann–Whitney test, except for gender and suppuration, which were analysed by the Chi-square test. The differences in bacterial numbers between the two diagnosis groups was also statistically analysed by the Mann–Whitney test, comparing the medians of the groups. The difference in prevalence of the studied taxa between the two clinical groups was analysed by the Chi-square test. The correlation between bacterial numbers and clinical parameters was investigated by Spearman correlation analysis. Differences were considered statistically significant at $P < 0.05$.

Results

Clinical and demographic characteristics of the subjects

A total of 43 samples from sites/patients with peri-implantitis and 41 samples from sites/individuals with peri-implant health were collected. Clinical measurements for one individual with peri-implant health were not available, and hence were not included in the corresponding analyses (Table 2). There were no differences in age and gender distribution between the two clinical groups. When the clinical parameters were considered on the implant level, PPD, PI, BOP and bone loss were significantly higher in the peri-implantitis, than peri-implant health group. On the contrary, the width of the keratinized mucosa was significantly lower in peri-implantitis, compared to peri-implant health. Although, the difference in implant age between the two groups was marginally above significance levels ($P = 0.055$), this was numerically higher in peri-implantitis compared to peri-implant health by approximately 1.7 years.

Estimation of total bacterial numbers

The total bacterial numbers were estimated using an oligonucleotide probe that identifies most oral eubacteria. Submucosal biofilm samples from sites with peri-implantitis exhibited higher total bacterial counts ($5.69 \times 10^6 \pm 1.32 \times 10^7$) compared to healthy sites ($3.37 \times 10^5 \pm 1.36 \times 10^6$), a difference that proved to be highly statistically significant ($P < 0.0001$).

Prevalence of *Synergistetes* and *Spirochaetes* in submucosal biofilm

The prevalence of the various *Synergistetes* and *Spirochaetes* clusters was thereafter investigated (Table 3). *Synergistetes* cluster A was detected in 51.2 % of the healthy sites and 81.4 % of the sites with peri-implantitis, a difference that proved to be significant. When the sub-clusters A1 and A2 were considered, the former was detected in 41.4 % of healthy and 74.4 % of peri-implantitis sites, whereas the latter in 48.7 % and 81.4 % of the sites, respectively. In both cases the difference was significant. In the case of *Spirochaetes*, *Treponema* cluster 1 (groups I-III) was detected in 17.0 % of healthy sites and 65.1 % of peri-implantitis sites, which proved to be significant. However, *Treponema* group IV was detected in only 2.4 % of the healthy, and 13.9 % of the peri-implantitis sites, an increase that was not significant. Hence, due to the low detection frequency and levels, no further statistical analysis was performed on the numbers of these bacterial taxa.

Numbers of *Synergistetes* and *Spirochaetes* in submucosal biofilm

The bacterial numbers of *Synergistetes* cluster A, their sub-clusters A1 and A2, as well as *Treponema* cluster 1 (groups I-III), in the submucosal biofilm samples were further evaluated. The data demonstrates that *Synergistetes* cluster A are found in 2.5-

log higher numbers at sites with peri-implantitis, compared to healthy sites (Figure 1A). Their distribution within *Synergistetes* sub-clusters A1 and A2 was further considered. Sub-clusters A1 and A2 were detected both at 2.8-log higher levels in peri-implantitis, compared to health (Figures 1B and 1C). Finally, in the case of *Treponema* cluster 1 (groups I-III), their numbers in peri-implantitis were higher than in peri-implant health by approximately 2.4-log (Figure 1D). The differences between the two diagnosis groups proved to be significant for all studied bacterial taxa ($P < 0.0001$). The median values, as well as lower and upper quartiles related to this Figure are provided in Table 4.

Correlation of microbiological and clinical parameters

Finally, the correlation between the bacterial numbers in submucosal biofilms and the clinical parameters of the corresponding sampled sites was investigated (Table 5). No correlation was found between implant age and the numbers of the different bacterial taxa in the biofilms. PPD positively correlated with the numbers of all studied taxa. PI correlated only with total bacterial numbers, but not with any of the specific taxa studied. BOP, bone loss and suppuration correlated with the numbers of *Synergistetes* cluster A and its sub-clusters, but not with *Treponema* cluster 1 (groups I-III) (correlation coefficients provided in Table 5).

Discussion

The present study investigated the prevalence and numbers of the bacterial phyla *Synergistetes* and *Spirochaetes* in submucosal biofilms from sites with peri-implantitis, or peri-implant health. Whereas the association of *Spirochaetes* with peri-implantitis has been established in earlier studies (Papaioannou, et al. 1995, Renvert,

et al. 2007), there has only been circumstantial evidence on the involvement of *Synergistetes* by sequencing approaches (Koyanagi, et al. 2010, Maruyama, et al. 2014). Hence, this is the first study to use a targeted approach in order to investigate the involvement of this new phylum in peri-implantitis. *Synergistetes* cluster A was detected in half of the healthy sites, and its prevalence was significantly increased by a further 30% in peri-implantitis. Absolute bacterial numbers of this cluster in biofilms were also profoundly increased in peri-implantitis by 2.5-log, in relation to peri-implant health. These data show a strong association of *Synergistetes* cluster A with peri-implantitis, which is well in agreement with the elevated numbers of *Synergistetes* cluster A in periodontitis, compared to periodontal health (Belibasakis, et al. 2013), or in NUG, compared to plaque-induced gingivitis (Baumgartner, et al. 2012).

A recent study analysing the prevalence and microbial identity of biofilm samples from sites with peri-implantitis by using a 16S rRNA gene clone library technique, supported that the phylum *Synergistetes* is, among other phyla, present only in peri-implantitis, and not in peri-implant health or periodontitis (Koyanagi, et al. 2010). A broad metagenomic approach has not been utilized in the present study, preventing the estimation of the relative role of *Synergistetes* within the whole microbial community. Nevertheless, the FISH method used here is a 16S rRNA-targeted approach that enabled with high specificity the identification and quantification of viable *Synergistetes* in both peri-implant health and disease. It should also be noted that this clinical microbiological study is a retrospective one. Hence, a limitation in the interpretation of the findings is that they do not necessarily imply a cause-effect relationship between *Synergistetes* and peri-implantitis.

The *Synergistetes* sub-clusters A1 and A2 were further considered, and their numbers were found to be equally elevated in peri-implantitis, compared to peri-implant health. In agreement with this finding, a similar increase of these two sub-clusters has been earlier demonstrated in NUG, compared to plaque-induced gingivitis (Baumgartner, et al. 2012), which may imply that the distribution of *Synergistetes* A sub-clusters may not be crucial factor in the etiology of oral infectious disease.

This study also investigated the association of *Spirochaetes*, represented by *Treponema* cluster 1 (groups I-III) and *Treponema* group IV, with peri-implantitis. Interestingly, the *Treponema* group IV was only rarely detected in peri-implantitis, or peri-implant health, and its prevalence did not significantly increase in the disease. In contrast, *Treponema* cluster 1 (groups I-III) was detected infrequently in peri-implant health, but its prevalence in peri-implantitis was significantly increased by almost 4-fold. Its absolute numbers in the biofilms were accordingly increased by 2.4-log, indicating a strong association with peri-implantitis. The increased presence of *Treponema* cluster 1 (groups I-III), particularly *Treponema denticola*, in peri-implantitis may not be a novel finding, as this has been already demonstrated by earlier works (Hultin, et al. 2002, Papaioannou, et al. 1995, Persson & Renvert 2014, Persson, et al. 2006, Renvert, et al. 2007, Shibli, et al. 2008). Accordingly, more recent metagenomic analysis document the broader presence of genera *Treponema* in peri-implantitis-associated biofilms (Koyanagi, et al. 2013, Koyanagi, et al. 2010, Kumar, et al. 2012, Maruyama, et al. 2014). The present study complements these earlier ones, by employing a 16S rRNA-targeted FISH method to identify and quantify their presence in peri-implantitis. Of note, while analysis of the microbial profiles according to smoking status was not performed here, this is an important factor to be considered, as smoking influences distinctively ecological succession in

biofilms and hence the composition of peri-implant microbiomes (Tsigarida, et al. 2015).

The association of clinical peri-implant parameters with the numbers of the studied taxa was further considered. The levels of *Treponema* cluster 1 (groups I-III) positively correlated with PPD, in line with earlier studies showing a correlation between increased levels of spirochetes and PPD in peri-implantitis (Papaioannou, et al. 1995, Renvert, et al. 2007). The present study failed, however, to disclose a significant correlation between *Treponema* cluster 1 (groups I-III) and any other clinical parameters, which may be at odds with a recent study showing the correlation of an uncultured *Treponema* sp. with several clinical peri-implant parameters (Maruyama, et al. 2014). As the methodological approach between the two studies is substantially different, it is difficult to compare the extent of overlap in the detected bacterial phylotypes. On the other hand, the levels of *Synergistetes* cluster A (including sub-clusters A1 and A2) in biofilms significantly correlated not only with PPD, but also with bone loss, suppuration and BOP, potentially indicating its stronger association with the severity of peri-implantitis, than *Spirochaetes*.

In conclusion, the present study shows that *Treponema* cluster 1 (groups I-III) and *Synergistetes* cluster A are highly associated with peri-implantitis, as indicated by their increased prevalence and numbers in the disease. On the contrary *Treponema* group IV displays only little association, whereas there are no distinctive differences between the two *Synergistetes* A sub-clusters. Moreover, *Synergistetes* cluster A is more strongly associated with the severity peri-implantitis than *Spirochaetes*. Collectively, the clinical implication of this study is the demonstration that *Synergistetes* are involved in peri-implantitis and may constitute a more suitable microbiological indicator for disease diagnosis than *Spirochaetes*.

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Tables

Table 1. 16 rRNA-targeted probe sequences, target taxa and validation references

<i>Probe</i>	<i>Sequence (5'–3')</i>	<i>Target taxa</i>
<i>EUB338-Cy3</i> (Amman <i>et al.</i> 1995)	GCTGCCTCCCGTAGGAGT	Most eubacteria
<i>SYN-A1409-FAM</i> (Zijngel <i>et al.</i> 2010, Baumgartner <i>et al.</i> 2012)	ACACCCGGCTCGGGTGGT	<i>Synergistetes</i> cluster A
<i>SYN-A1-632-FAM</i> (Baumgartner <i>et al.</i> 2012)	GCACCTCAGTCTCAACTGC	<i>Synergistetes</i> cluster A1
<i>SYN-A2-207-Cy3</i> (Baumgartner <i>et al.</i> 2012)	CCTCCTCCAGCGCATCTC	<i>Synergistetes</i> cluster A2
<i>TrepG1-679-Cy3</i> (Zijngel <i>et al.</i> 2010)	GATTCCACCCCTACACTT	<i>Treponema</i> groups I,II,III
<i>Tlema738-Cy3</i> (Guggenheim <i>et al.</i> 2009)	GCGTCAATTATCTGCCGG	<i>Treponema</i> group IV

Table 2. Patient demographic data and clinical parameters of the sampled implants

<i>Parameter</i>	<i>Health</i>	<i>Peri-implantitis</i>	<i>P value</i>
Patient age (years + SD)	57.6 ± 15.1	59.1 ± 13.0	0.724
Gender (M/F)	13/28	14/29	0.934
Implant age (years + SD)	6.1 ± 4.5	7.8 ± 4.8	0.055
Bone loss (mm)	0.02 ± 0.1	6.3 ± 2.6	<0.0001 *
Suppuration (+/total)	0/40	22/43 (51.2 %)	<0.0001 *
PPD (mm)	3.0 ± 0.4	6.8 ± 2.3	<0.0001 *
BOP (% sites)	23 ± 28	79 ± 29	<0.0001 *
PI (% sites)	15 ± 25	40 ± 37	0.002 *
Keratinized mucosa (mm)	3.2 ± 1.5	1.9 ± 1.7	<0.0001 *

For patient age and gender, n=41 for peri-implant health and n=43 for peri-implantitis. For the remainder clinical measurements n=40 for peri-implant health and n=43 for peri-implantitis. * Statistically significant ($P<0.05$) difference between the two clinical diagnosis groups

Table 3. Prevalence of *Synergistetes* and *Spriochaetes* among subjects with peri-implantitis and peri-implant health.

<i>Cluster/group</i>	<i>Health</i>	<i>Peri-implantitis</i>	<i>P value</i>
<i>Synergistetes</i> A	21/41 (51.4 %)	35/43 (81.4 %)	0.003 *
<i>Synergistetes</i> A1	17/41 (41.4 %)	32/43 (74.4 %)	0.002 *
<i>Synergistetes</i> A2	20/41 (48.7 %)	35/43 (81.4 %)	0.001 *
<i>Treponema</i> cluster 1	7/41 (17.0 %)	28/43 (65.1 %)	< 0.001 *
<i>Treponema</i> group IV	1/41 (2.4 %)	6/43 (13.9 %)	0.056

* Statistically significant ($P<0.05$) difference between the two clinical diagnosis groups.

Table 4. Median and (lower/upper) quartile values of *Synergistetes* and *Spriochaetes* in the peri-implantitis and peri-implant health groups

<i>Cluster/group</i>	<i>Health</i>	<i>Peri-implantitis</i>
<i>Synergistetes</i> A	50 (25/250)	2.6×10^3 (250/4.4x10 ⁴)
<i>Synergistetes</i> A1	25 (25/130)	300 (25/2.3x10 ⁴)
<i>Synergistetes</i> A2	25 (25/100)	400 (50/1.8x10 ⁴)
<i>Treponema</i> cluster 1	25 (25/25)	250 (25/1x10 ⁴)

Table 5. Correlation between bacterial numbers in submucosal biofilms and clinical parameters of the samples sites.

<i>Parameter</i>	<i>Total</i>	<i>SynA</i>	<i>SynA1</i>	<i>SynA2</i>	<i>Trep1</i>
PPD	0.39 *	0.53 *	0.51 *	0.58 *	0.34 *
BOP	0.20	0.39 *	0.35 *	0.39 *	0.24
PI	0.25 *	0.23	0.12	0.07	0.14
Suppuration	0.23 *	0.43 *	0.33 *	0.49 *	0.06
Implant wear	0.05	0.20	0.27	0.15	0.20
Bone loss	0.45 *	0.62 *	0.47 *	0.53 *	0.34

The Spearman's r correlation coefficient is provided. * Statistically significant correlation ($P < 0.05$). Total; total bacterial numbers, SynA; *Synergistetes* cluster A, SynA1; *Synergistetes* sub-cluster A1, SynA2; *Synergistetes* sub-cluster A2, Trep1; *Treponema* cluster 1 (groups I-III).

Figure legends

Figure 1

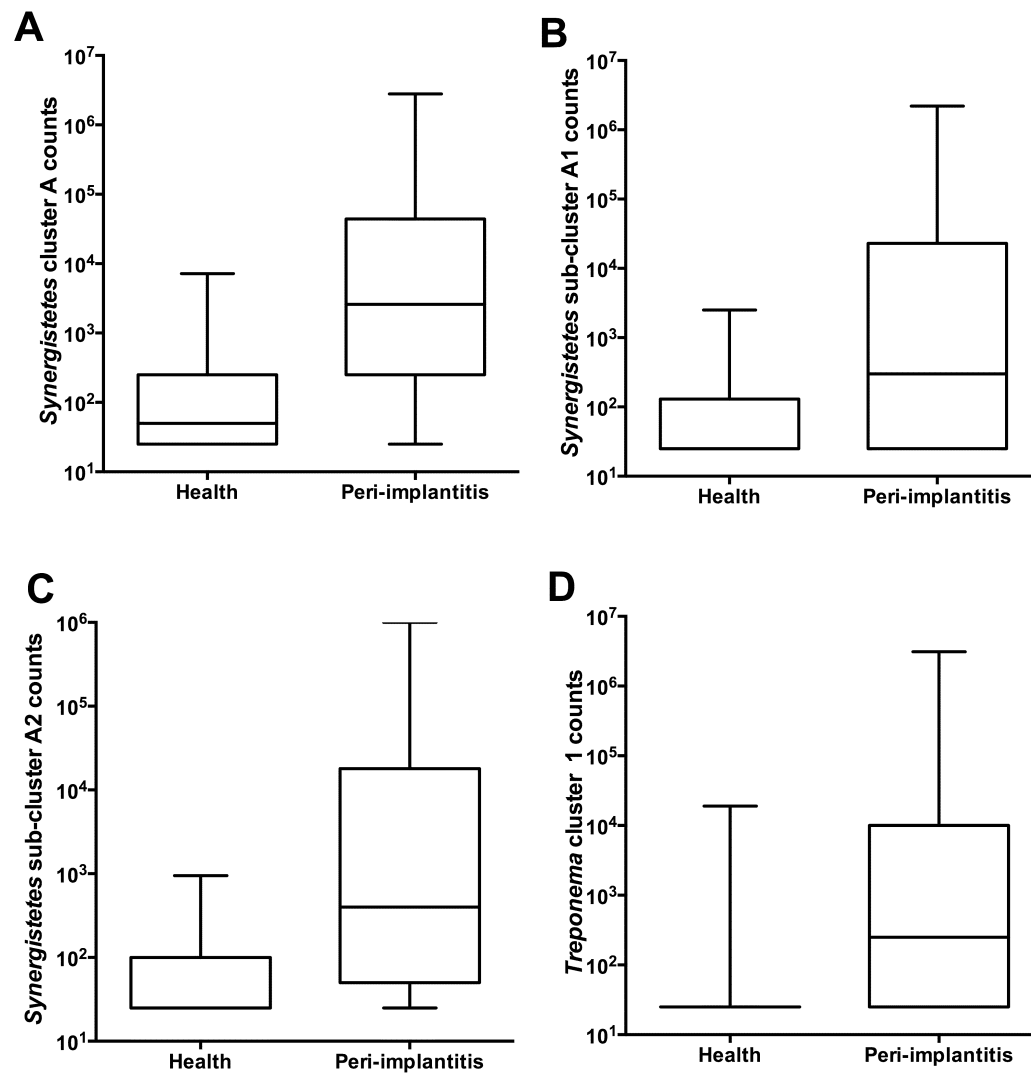


Figure 1. Numbers of *Synergistetes* cluster A (A), *Synergistetes* sub-cluster A1 (B), *Synergistetes* sub-cluster A2 (C), and *Treponema* cluster 1 (groups I-III) (D) in submucosal biofilms of patients with peri-implantitis (n=43) and peri-implant health (n=41). The numbers represent the positive bacterial counts by FISH, per sample. The differences between all pair-wise comparisons proved to be statistically significant ($P<0.05$).